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PNAS 1990;87;4580-4584
doi:10.1073/pnas.87.12.4580

Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4580–4584, June 1990
Genetics

Direct electrophoretic detection of the allelic state of single DNA molecules in human sperm by using the polymerase chain reaction

(genetic recombination/linkage/sperm typing/genetic disease diagnosis/allele-specific amplification)

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Communicated by Walter M. Fitch, March 1, 1990 (received for review December 10, 1989)

ABSTRACT **We have developed a procedure that allows the detection of polymerase chain reaction (PCR) products derived from a single target DNA molecule in a human sperm without using radioactive probes. With this method, three genetic loci present in a single sperm can be amplified simultaneously. The amplification procedure is specific as well as efficient and permits detection of the PCR product by ethidium bromide staining after polyacrylamide gel electrophoresis. When allele-specific PCR primers that differ in length are used, the size of the PCR products of different alleles also vary in length, allowing the allelic state at each locus to be determined electrophoretically. Studies on individual sperm by using this procedure should facilitate the measurement of genetic recombination in humans over small physical distances. The ability to directly analyze the allelic state of PCR products from one cell rapidly and simply will also be useful for the prenatal diagnosis of genetic disease, especially in the analysis of single blastomeres taken from *in vitro* fertilized eggs prior to implantation.**

Recently, an approach to measuring human genetic recombination between polymorphic DNA markers has been developed that is based upon the analysis of DNA sequences present in single sperm (1, 2). The polymorphic regions at two or three loci in a single sperm are first amplified to detectable levels by the polymerase chain reaction (PCR; refs. 3–5). The allelic status at each locus is determined by hybridizing the PCR products to allele-specific oligonucleotide (ASO) probes (6). In this way the genotype of an individual sperm can be identified as recombinant or nonrecombinant. This sperm typing procedure makes it possible to analyze a large number of individual meiotic products. Thus the frequency of recombination between closely linked human polymorphic DNA markers can be determined with a statistical accuracy far greater than conventional family studies allow (2, 7). Strategies for making a genetic map by ordering a set of DNA markers on a chromosome by three-point crosses using the sperm typing approach have also been put forward (7, 8).

Constructing a genetic map with high resolution requires a large sample size to ensure statistical reliability and would benefit from methods that facilitate the automation of the sperm typing procedure. In this paper we present a method for analyzing the allelic status of the PCR products of single sperm. Instead of using dot-blot hybridization with ASO probes we distinguish between alleles by directly examining the sizes of the PCR products after polyacrylamide gel electrophoresis and ethidium bromide staining. This single molecule detection system uses allele-specific PCR primers with different lengths in combination with a strategy we call "heminesting." Since the PCR products amplified from different alleles by this method have different lengths, we call our procedure "allele discrimination by primer length" or ADPL. The ease with which allelic status can be determined suggests that this method could also prove valuable for prenatal diagnosis of genetic disease.

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MATERIALS AND METHODS

Polymorphic Markers, PCR and ADPL Primers, and ASO Probes. The restriction fragment length polymorphisms (RFLPs) at the parathyroid hormone (PTH), $^{G}\gamma$-globin ($^{G}\gamma$), and low density lipoprotein receptor (LDLr) loci (human gene nomenclature symbols *PTH*, *HBG2*, and *LDLR*, respectively) were described previously (1, 2). The sequences of the PCR and ADP primers and ASO probes used for these loci are listed in Table 1. The locations of the primers and the lengths of the target fragments generated from each corresponding primer pair are diagrammed in Fig. 1.

PCR Conditions. *Amplification of single sperm sequences by using heminested primers.* Sperm were purified and single sperm samples were prepared by using the procedure described by Cui *et al.* (2). Three different protocols for amplifying the LDLr locus are compared. *Method 1:* Method 1 was the original procedure described by Li *et al.* (1), which involved lysis by a solution with detergent, reducing agent, and proteinase K, 20 cycles of co-amplification of the LDLr and DQα genes (in the human histocompatibility complex), followed by 50 cycles of additional amplification of an aliquot with only LDLr primers LrM1 and LrM4. *Method 2:* After alkaline lysis (2) each neutralized sperm sample (approximate volume 5–6 μl) was brought up to a volume of 50 μl with PCR reagents. The final mix contained 1× PCR buffer (2), 100 μM each dNTP, 0.1 μM each of the primers for LDLr (LrM1, LrM4) and $^{G}\gamma$ (GgM1, GgM2), 0.05 μM each of the primers for PTH (TaM2, TaM3), and 1 unit (as defined by the supplier, Perkin–Elmer/Cetus) of *Thermus aquaticus* (*Taq*) DNA polymerase. Forty PCR cycles with 95°C for 15 sec for denaturation and 60°C for annealing and extension were carried out. The time at 60°C was 3 min for the first 10 cycles and 2 min for the remaining 30 cycles. After the first round of 40 amplification cycles, a 1-μl aliquot from each reaction was added to 50 μl of PCR solution in a reaction tube containing 1× PCR buffer, 50 μM each dNTP, 0.2 μM LDLr primers LrM1 and LrM4, and 1 unit of *Taq* DNA polymerase. The samples were amplified for an additional 25 cycles at 95°C for 15 sec for denaturation and 15 sec at 54°C for annealing and extension. *Method 3:* Method 3 was the same as method 2, except that in the 25-cycle secondary round of amplification one of the initial primers (LrM4) was replaced by one primer (LrM6) internal to the original two.

Abbreviations: PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; ADPL, allele discrimination by primer length; RFLP, restriction fragment length polymorphism; PTH, parathyroid hormone; $^{G}\gamma$, $^{G}\gamma$-globin; LDLr, low density lipoprotein receptor.

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Table 1. Oligonucleotide primers and probes

Locus	Name	Sequence (5' to 3')
PTH	TaM2	GATCTCTTCCTGGGAAGAAG
	TaM3	GATACCTGCAAAAGACATGG
	TaM5	TCCCATTAGCTCCCCACTTC
	TaM6	actggaactacagatTCCCATTAGCTCCCCACTTT
	TaM4	GAGAAACAGAGAGGGCCACT
	TaBW	TCCCCACTTCGAAATGATA
	TaBM	TCCCCACTTTGAAATGATA
$^{G}\gamma$	GgM1	AGTGACTAGTGCTGCAAGAA
	GgM2	CTCTGCATCATGGGCAGTGA
	GgM3	GCCTCCAGATAACTACACACCC
	GgM4	gaatcatagtaacatGCCTCCAGATAACTACACA-CCA
	GgM6	TGGTATCTGGAGGACAGGGCACTGGCCAC
	GgBW	TTCTGGGTGGAAGCTTGGT
	GgBM	TTCTGGGTGGAAGCTGGGT
LDLr	LrM1	AGTGCCAACCGCCTCACAGG
	LrM4	CCTCTCACACCAGTTCACTC
	LrM7	GGGTGAGGTTGTGGAAGAGG
	LrM8	atttgcagagacataGGGTGAGGTTGTGGAAGAGA
	LrM6	TGGCTGGGTGAGGTTGTGGA
	LrBW	AGGATATGGTCCTCTTCCA
	LrBM	TGGAAGAGAACCATATCCT

Nucleotides in lowercase letters were added to allow discrimination of PCR products of different alleles. BW or BM is included in the names of the hybridization probes.

Allelic discrimination of PCR products from single sperm. This procedure also involved a two-step amplification. The first step was the same as method 2; this step was followed by a second round of amplification. Six 1-μl aliquots were taken from each reaction mixture. The ADPL procedure was carried out on three of them. For each of the three loci to be studied by ADPL (PTH, $^{G}\gamma$, and LDLr), the aliquot was added to 50 μl of PCR solution in a separate reaction tube. Each reaction mixture had a final concentration of 1× PCR buffer, 4 μM each dNTP (2 μM of it resulted from carry-over by the 1-μl aliquot), and 1 unit of *Taq* DNA polymerase. Three primers were added. The appropriate regular primer and short allele-specific primer were at 2 μM each. The concentration of the appropriate long allele-specific primer was locus dependent (0.1 μM TaM6, 0.4 μM GgM4, or 0.5 μM LrM8). Each sample was amplified for 25 cycles with a 95°C 15-sec denaturation and 15 sec at 65°C for annealing and extension.

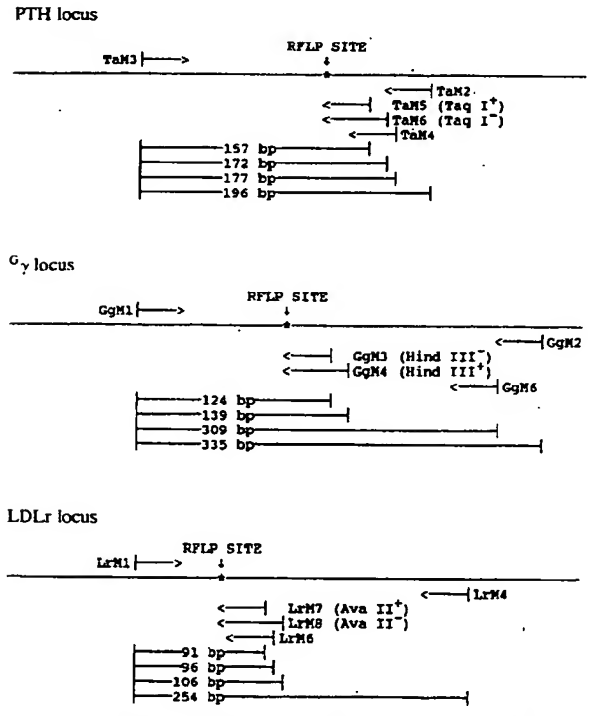

FIG. 1. Locations of the PCR primers and lengths (in base pairs, bp) of the PCR products. The restriction enzyme that can distinguish between alleles at the polymorphic site is shown adjacent to the allele-specific primers at each locus.

The remaining three aliquots from the first round of PCR on each sperm sample were used to test the reliability of the ADPL procedure. In these experiments, the two allele-specific primers for each locus in the ADPL procedure were replaced by a single primer that was internal to the two original primers: TaM4 for PTH, GgM6 for $^{G}\gamma$, and LrM6 for LDLr. For these secondary reactions the protocol was the same as in the ADPL experiments with the following differences: we used 50 μM of each dNTP and 0.2 μM of each primer. The temperature for annealing and extension was 54°C for PTH and LDLr and 65°C for $^{G}\gamma$. Following amplification these samples were used for ASO hybridization analysis with the probes shown in Table 1.

Semen samples. Three microliters of semen was washed once with 50 μl of water and spun at maximal speed in a Biofuge A microcentrifuge (Heraeus) to pellet the sperm. The single sperm alkaline lysis procedure (2) was used except that the total volume of the lysis and neutralization solution was 50 μl. For each locus a 10-μl aliquot was directly subjected to the ADPL procedure without a preliminary amplification round. For each locus the concentration of reagents was identical to that used for the single sperm ADPL step. However, instead of 25, 30 PCR cycles were used. The genotype of each semen sample was independently confirmed by carrying out PCR amplification and ASO hybridization using the regular primers.

Analysis of the PCR Products. As mentioned above, three aliquots were taken from each sperm sample for ADPL after the initial rounds of amplification, one aliquot for each locus. To examine the genotype of the original sperm with respect to all three loci by using ADPL, 5-μl portions from the three separate ADPL products were mixed with 10 μl of gel loading buffer (0.25% bromophenol blue and 15% Ficoll type 400). The sample was loaded on an 8% polyacrylamide gel (10 cm × 8 cm × 0.15 cm) and subjected to electrophoresis at 100 V for 1.5 hr and viewed under UV illumination after staining with ethidium bromide. The same method was used for the semen samples.

Dot-blot and ASO hybridization were performed as before (1, 2). Electroblotting to a nylon filter after gel electrophoresis was carried out by the procedure of Pauli *et al.* (9) except that an 8% polyacrylamide (polyacrylamide/bisacrylamide = 19:1) gel was used. After the transfer the filter was treated and hybridized to the LDLr probe as described in ref. 1.

RESULTS

Ethidium Bromide Detection of PCR Products from Single Sperm on Polyacrylamide Gels. When the LDLr locus in single sperm samples is amplified 20 cycles in the first round and 50 cycles in the second round (method 1) and the products studied by gel electrophoresis, a number of background bands in addition to a smear are observed (Fig. 2, lanes 1–3). Lanes 4–6 in Fig. 2 show the results of using method 2, which consists of 40 primary cycles and 25 secondary ones. In this experiment background was significantly reduced but not eliminated. Finally, lanes 7–9 show the result of carrying out the same procedure used in lanes

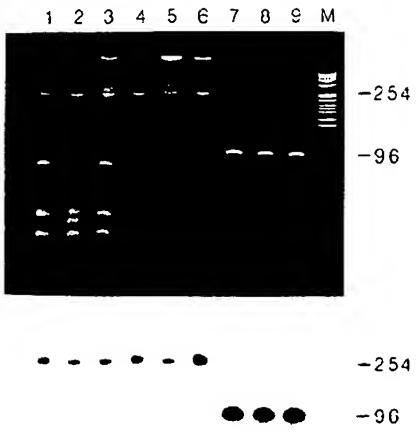

FIG. 2. Comparison of PCR protocols on the efficiency of single sperm PCR as detected by gel electrophoresis. (*Upper*) Staining the gel with ethidium bromide. (*Lower*) Electroblotting the gel and hybridizing with a radioactive LDLr probe. Lanes 1–3, LDLr products amplified with the original procedure (1). The co-amplified locus in the initial rounds of PCR was HLA DQα. Lanes 4–6, PCR products generated by method 2. The co-amplified loci in the initial rounds of PCR were PTH and $^{G}\gamma$. Lanes 7–9, same as in lanes 4–6, but a heminested primer pair (LrM1 and LrM6) was used in the second round of PCR. Lane M, size markers from pBR322 digested with *Msp* I. The sizes of the LDLr PCR products (in bp) are shown.

4–6 except that the second round of amplification on the aliquot involved replacement of one of the original primers with an internal primer, a procedure we call heminesting. Heminesting is a modification of the "nesting" method (5, 10), in which a second round of amplification uses two new amplification primers specific for the same target, both of which are internal to the first primer pair. While the introduction of the *Taq* DNA polymerase eliminates the need for nesting in most PCR applications, we find heminesting extremely valuable for the analysis of single cells. As lanes 7–9 in Fig. 2 show, there is more product made with the heminesting procedure and the background is virtually eliminated. The electrotransfer (9) and hybridization experiment shown in the lower half of the figure, in which an LDLr-specific hybridization probe was used, confirms that the major ethidium bromide stained bands are the expected product.

Distinguishing Between Allelic Variants by the Size of the PCR Product: ADPL. We designed a system to allow us to distinguish between alleles at a locus on the basis of the size of the PCR product itself. If two alleles differ by a single base substitution, two PCR primers can be designed to contain the variable site at their 3′ ends. Thus each primer is identical to one allele and differs from the other by a single base substitution at its 3′ end. It is expected that under the appropriate conditions the extension of the completely matched primer will be more efficient than the extension of the primer with a mismatch at the 3′ end. To distinguish between the PCR products from the two alleles we constructed the two allele-specific primers so that they differed in length by 15 nucleotides and therefore their products also were of different sizes and could be distinguished by gel electrophoresis. The lengths of the final PCR products from different loci were also taken into consideration when designing the primers so that all the products from three loci were properly spaced within a single lane after polyacrylamide gel electrophoresis. The addition of 15 nucleotides to one ADPL primer affects the efficiency of amplification. As a consequence we had to change the relative concentrations of the two allele-specific primers from the usual 1:1 to: 1:20 for the PTH locus, 1:5 for the $^{G}\gamma$ locus, and 1:4 for the LDLr locus.

We attempted to analyze three independent genetic loci simultaneously from a single sperm sample by using a direct gel electrophoresis detection system. To minimize background generated by the large number of PCR cycles required when using single sperm as starting material, we combined the ADPL concept with a heminesting protocol. The initial rounds of amplification contained three primer pairs: one pair each for the PTH, $^{G}\gamma$, and LDLr loci. Following this, three aliquots from the PCR mixture were taken. Each was further amplified by using heminesting with one regular and two allele-specific primers for one of the three loci. The expected sizes of the allele-specific products are shown in Fig. 1.

Using the procedure described above, we typed 88 single sperm samples from an individual heterozygous at all three loci along with 11 negative controls. Fig. 3 shows some typical results. Lane 9 shows the position of all six allele-specific fragments amplified from a semen sample from the triply heterozygous donor by using the ADPL procedure. Since each sperm is haploid, each lane is expected to have only three bands, representing one allele from each locus.

In addition to the three aliquots taken from the initial round of amplification of each sperm sample to carry out ADPL, an additional three aliquots from the same sample were used to

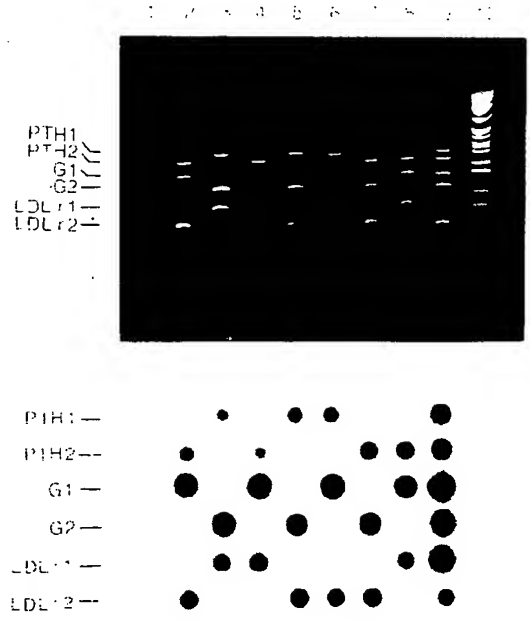

FIG. 3. Determination of the allelic status at three loci in single sperm by using the ADPL procedure. The sizes of each allelic product are shown in Fig. 1. (*Upper*) Ethidium bromide staining. (*Lower*) Parallel experiment using the ASO hybridization approach to confirm the ADPL allele-typing results. Lane 1, Negative PCR control, which received all reagents except a sperm. PTH1 and -2 are the alleles at the PTH locus, lacking and containing the *Taq* I recognition sequence, respectively (termed *Taq* I^- and *Taq* I^+, respectively). G1 and -2 are the *Hin*dIII$^+$ and *Hin*dIII$^-$ alleles at the $^{G}\gamma$ locus. LDLr1 and -2 are the *Ava* II^- and *Ava* II^+ alleles at the LDLr locus. Lanes 2–8, single sperm samples. Lane 9, ADPL products from a 3-μl sample of semen from the triply heterozygous sperm donor. Lane 10, pBR322 digested with *Msp* I. The sequences of the hybridization probes are shown in Table 1.

confirm the reliability of the ADPL procedure by using ASO hybridization probes. To accomplish this goal each of these aliquots was subjected to a secondary round of amplification in which the two allele-specific primers were replaced by one heminesting primer that was not allele-specific but generated instead a target fragment containing the polymorphic site. The allelic status at each of the loci was then determined by using ASO hybridization probes. Fig. 3 *Lower* shows the results obtained for these selected samples. In these seven cases and in the remaining 81 of 88 samples (data not shown) the ADPL and ASO procedures gave identical results. All 11 blanks showed no signal regardless of the detection method used.

Application of ADPL to Semen Samples. For each locus to be tested, 10 μl of the lysed sample (equivalent to 0.6 μl of semen and containing approximately 5×10^4 sperm) was directly subjected to the ADPL procedure without the initial amplification step. As discussed above, parallel experiments were carried out to confirm the ADPL results by using an ASO hybridization assay. The results are shown in Fig. 4 *Lower*. Among 12 semen samples, 11 showed an exact correspondence between the two typing methods. The sample in lane 6 was typed as heterozygous at the $^G\gamma$ locus by using ADPL, but by ASO analysis it was clearly homozygous for the *Hin*dIII$^+$ allele. Repeat experiments gave the same result. We independently assessed the allelic state at this locus, using the fact that the two alleles can be distinguished by restriction enzyme digestion. The sample was amplified at the $^G\gamma$ locus, using primers GgM1 and GgM2 for 30 cycles. Along with appropriate controls to account for the degree of digestion, the 335-bp PCR product was digested with *Hin*dIII. The results are shown in Fig. 5. The sample in question produced two bands that are characteristic of the *Hin*dIII$^+$ allele (102 bp + 233 bp) and a 335-bp band characteristic of the *Hin*dIII$^-$ allele and confirmed the ADPL results. One possible explanation is that, in addition to having the restriction enzyme polymorphism at this site, this individual is heterozygous for another base substitution that is in the region recognized by the ASO probes but outside the restriction enzyme site. The additional substitution could result in a complete failure of hybridization with one of the ASO probes without affecting allele-specific amplification.

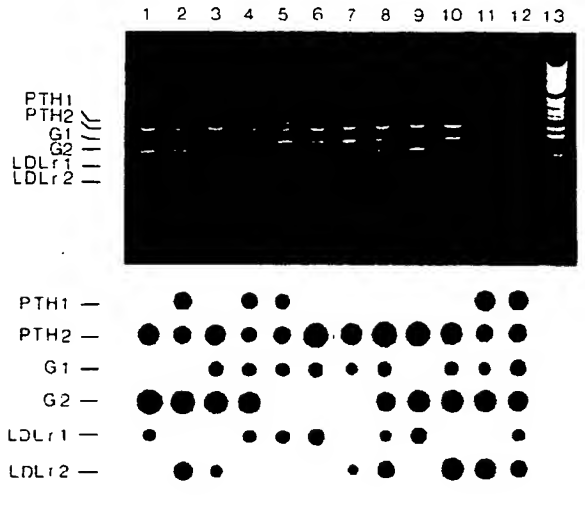

FIG. 4. Determination of the genotype of human individuals at three genetic loci by using ADPL on semen samples. (*Upper*) Ethidium bromide staining. (*Lower*) Confirmatory results from ASO hybridization. Lanes 1–12 contain the ADPL products for the PTH, $^G\gamma$, and LDLr loci from different semen samples. Lane 13, *Msp* I digest of pBR322. Note: An inconsistent result is observed at the $^G\gamma$ locus in the sample in lane 6 and is discussed in the text and studied in Fig. 5.

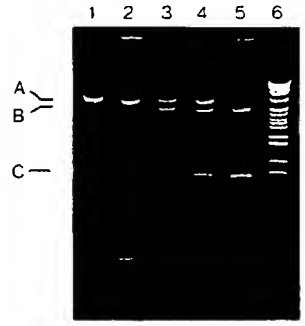

FIG. 5. Restriction enzyme characterization of the polymorphism at the $^G\gamma$ locus in the sample from lane 6 of Fig. 4. Three microliters of semen sample was amplified for 30 cycles with GaM1 and GaM2, generating a 335-bp fragment. Ten units of *Hin*dIII restriction enzyme was added to 10 μl of the PCR product from each sample with an adjustment of the Mg^{2+} concentration to 10 mM. The whole digest was subjected to polyacrylamide gel electrophoresis. Fragment A, undigested PCR product. Fragments B and C, the two bands resulting from cleavage of the PCR product at the *Hin*dIII site. Lane 1, digested sample from a control individual with a −/− genotype; lane 2, undigested sample from the individual in question; lane 3, digested sample from the individual in question; lane 4, digested sample from a control individual with a +/− genotype; lane 5, digested sample from a control individual with a +/+ genotype; lane 6, *Msp* 1 digest of pBR322.

DISCUSSION

In a single sperm, each chromosome is normally represented by a single DNA molecule. To analyze a unique DNA sequence in a single sperm, the target must be amplified by a factor of at least 10^{11} to detect the specific target fragment with a radioactive probe. While radioactive detection of sequences amplified from single sperm has been accomplished (1, 2), analyses of the same samples by gel electrophoresis and ethidium bromide staining show a number of additional background bands, some of which may migrate at or very close to the position expected for the PCR product (Fig. 2 and data not shown). It is generally accepted that background fragments can result from two types of nonspecific amplification. One is the production of dimers of the primers. These "primer dimers" are usually one of the major PCR products after a large number of cycles (>35 with *Taq* DNA polymerase) have been carried out, since the initial formation of the first "primer dimer" is a low-probability event. Higher-order concatemers of the primer are also thought to form. Second, background fragments can arise due to rare nonspecific priming and amplification of nontarget genomic DNA segments. The lower the amount of starting DNA, the greater the number of PCR cycles required and therefore the greater the probability that these rare events will occur.

There would be many advantages to being able to reduce the background and unambiguously identify specific PCR products by gel electrophoresis after amplification of one or only a few molecules. By using a "full" nesting technique (5) the β-globin gene has been electrophoretically detected with little background in single mouse blastomeres; no signal was detected from eggs of mice with a globin gene deletion (10). By using allele-specific primers of different lengths in a heminesting protocol we not only eliminate background PCR products but are capable of analyzing the allelic state at three independent loci simultaneously in a single cell. Our procedure eliminates labeled probes required by the other proto-

cols. In addition it does not depend upon polymorphisms that alter cutting at a restriction enzyme site.

Allele-specific amplification requires that the thermal stability of the mismatched primer-template complex be reduced compared with the perfectly matched one and/or that the DNA polymerase be relatively inefficient in extending a primer if there is a mismatch at the 3′ end. Because *Taq* DNA polymerase lacks a 3′-to-5′ exonuclease activity (11) there is every reason to expect some inefficient extension of 3′-terminal mismatches. A number of different strategies employing these concepts have been used to achieve allele-specific amplification on relatively large amounts of DNA (12–15). In these applications, two aliquots of each sample are needed. Each aliquot is usually amplified with one common primer and one of the two alternative allele-specific primers. The allelic nature of the sample (+/+, −/−, or +/−) is deduced by observing which aliquots produce a PCR product. To achieve allele specificity these experiments require one or a combination of the following strategies: (*i*) very short primers (14 bases) and a high temperature at the annealing step; (*ii*) short (<16 bases) competitive primers with base substitution(s) in the middle, or (*iii*) addition of mismatches near and/or at the 3′ end of the primers. We achieved allele-specific PCR by using the method of Ehlen and Dubeau (14). They demonstrated that allele-specific amplification using PCR primers with a 3′-terminal mismatch could be significantly enhanced by lowering the total dNTP concentration commonly used by almost a factor of 100. This modification, which follows from what is known about the fundamental enzymology of mismatch extension (16), allows significant discrimination such that extension of the mismatched primer is greatly reduced even when both primers are present in the same reaction tube. We used 4 μM of each dNTP. In our hands 2 μM of each dNTP adversely affected the total PCR efficiency, while 10 μM seriously affected allele specificity (data not shown).

Beginning with a single target molecule, it is necessary to carry out PCR in two steps to allow the simultaneous analysis of two (1, 2) or three loci (this paper). We think this may be especially important in ADPL analysis, since we have shown that allele-specific discrimination gradually erodes as the number of PCR cycles increases. Thus, starting with a single target molecule, at least 40 cycles of preliminary amplification are necessary before beginning ADPL.

The ADPL procedure is a highly reliable method for sperm typing. The frequency of recombination between the PTH and $^{G}\gamma$ loci estimated by using ADPL with this limited number of samples was 15.5%, which compares favorably with our recent large-scale sperm typing estimate (16%) using ASO hybridization (2). We also estimated the typing efficiency for each locus among the 88 sperm samples by dividing the number of samples showing one of the two expected bands at a locus by the total number of samples. We excluded from this calculation the samples that showed no evidence for amplification at any of the three loci (9 samples) or samples that showed evidence of more than one allele at any of the loci (10 samples). The calculated efficiencies of detecting an allele if it were present were 94% for PTH, 91% for $^{G}\gamma$, and 89% for LDLr. These efficiencies are quite similar to the maximum likelihood estimates obtained by using the ASO detection method on over 700 samples (2).

ADPL may be valuable for the prenatal diagnosis of genetic diseases, especially on samples that contain limited amounts of DNA. It will be especially useful for preimplantation prenatal diagnosis carried out on eggs fertilized *in vitro*. In such studies a single diploid blastomere taken at a very early stage of development would be the target of DNA analysis. Model studies on single human blastomeres and oocytes have recently been carried out (17, 18). In the one experiment where a single-copy gene was amplified in human oocytes (18) background PCR products made it difficult to analyze the allelic state of the locus by restriction enzyme digestion. ADPL would be expected to improve diagnostic accuracy significantly and allow the simultaneous analysis of several loci on the same sample. Experiments on single cells necessarily require rigorous control of contamination. In our experiments using sperm, each cell is expected to contain only one of two alleles present in the donor's somatic tissue. Thus, our data provide the strongest kind of evidence that we can analyze the amplification products from a single DNA molecule and that our results are not confounded by contamination.

The high efficiency of this PCR procedure could also benefit forensic studies, which can require allelic typing on extremely small samples of semen or other tissues. Finally, DNA rearrangements that are characteristic of the differentiation of certain cell types, for example, B- and T-cell development, may be directly and efficiently studied at the single cell level by using this procedure.

We acknowledge the helpful discussions of Dr. Gino Cortopassi. We are especially grateful for the cooperation of Dr. Cappy Miles Rothman and dedication of Diane Vieira in the selection of normal semen samples. We also thank Cetus Corporation for the gift of a Perkin–Elmer/Cetus thermocycler. This research was supported in part by grants from the National Institute of General Medical Sciences.

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